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FOCUSING ON MORPHOLOGY: APPLICATIONS AND IMPLICATIONS OF CONFOCAL LASER SCANNING MICROSCOPY (DIPTERA: CAMPICHOETIDAE, CAMILLIDAE, DROSOPHILIDAE)

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Abstract.—Confocal laser scanning microsocopy (CLSM) produces high fidelity, three-dimensional digital images of complex and phylogenetically informative, diagnostic insect structures such as the male genitalia. This study examined terminalia, mouthparts, and antennae of five genera in three ephydroid fly families—Campichoetidae, Camillidae and Drosophilidae—by utilizing the natural fluorescence of insect tissues under visible light (543 nm) excitation. CLSM images better reveal the shapes and positions of individual structures, and particularly their connections, as compared to conventional bright field light microscopy where fine layers and sutures are often obscured. CLSM has important implications for imaging valuable insect specimens, including types, and obviates the need for re-examination.

Key Words: Ephydroidea, genitalia, systematics, pinned specimens

Though considered quaint by some, the 300-year tradition of studying pinned insect specimens is actually an ingenious method for preserving and observing a microscopic landscape of setae, spines, sensilla, appendages, and microsculpture *in situ*. Modern systematic revisions, for example, emphasize the diversity of characters and their phylogenetic reliability, so it is not unusual now to see hundreds of anatomical structures reported in any particular monograph, and the source is hardly exhausted (Grimaldi 1990, Grimaldi and Nguyen 1999, Mathis and Zatwarnicki 2002). New morphological details are even still being discovered on what is arguably the best known eukaryote, *Drosophila melanogaster* Meigen (Ashburner 1989). Here, we present confocal laser scanning microscopy (CLSM) as a powerful new tool in the arsenal of insect morphological studies.

Insect morphology is traditionally studied using light microscopy, both stereomicroscopy (for opaque and cleared specimens, generally 10–70×), and compound microscopy (for transparent specimens, generally 60–400×). The optical constraints of these two techniques complement each other: stereomicroscopy provides relief and three-dimensionality, while compound microscopy provides higher resolution and magnification. Both types of microscopes will continue to be instrumental for routine morphological analysis, since the investigator can quickly obtain information about a specimen. However, record-

ing images using either stereo or compound microscopy is beset with the classical problem of the interplay between resolution, illumination, and depth of field.

Scanning electron microscopy (SEM) is also routinely used in insect morphology, although the availability of these instruments is usually very limited. SEM provides unparalleled resolution and depth of field at high magnifications, but can only image surfaces. Insect structures are rarely just flat fields; they usually consist of folds, apodemes, and obscuring layers and lobes, such as in complex male genitalia, most of which are hidden from view in a typical SEM mount. Additionally, specimen preparation for conventional SEM can be destructive—samples must be coated with a thin metal layer (usually gold/palladium) to render the specimen electrically conductive. Alternative SEM technologies exist, such as variable pressure (or low vacuum) SEM (Sammons and Marquis 1997) and cold field-emission SEM (Klaus 2003), that allow for uncoated specimen viewing, however, limited surface views of exposed structures is still a problem with these instruments.

CLSM uses a conventional compound microscope setup, but utilizes laser light of specific wavelengths for specimen illumination rather than white light (for an introduction to CLSM, see Paddock 1999). The light used for illumination (excitation wavelength) excites fluorescent molecules present in the specimen, and filters are then used to isolate the specific wavelengths of light emitted by the excited molecules. Normally, fluorescence in CLSM depends on the fluorescent label or tag applied to the specimen; however, in the case of insects and other arthropods, the fluorescence is due to naturally occurring compound(s) (Neff et al. 2000, Lardeux et al. 2000). The real power of CLSM is the ability to "optically section" a specimen by placing a pinhole aperture in front of the final signal detector. The pinhole allows only the signal from the plane of focus to be collected, therefore excluding obscuring out-of-focus light. The plane of focus is changed in minute and equal increments by the software controlling the microscope. The optical sections can then be reconstructed into a three-dimensional (3-D) object, thus obtaining a clear image of the original structure without the blurring usually associated with images of thick objects obtained on a compound microscope. If the data from the specimen are collected under optimal conditions (see Klaus et al. 2003) a faithful 3-D rendering can be created and rotated in space for viewing at any angle. Thus, CLSM provides the resolution of compound light microscopy, combined with the relief and three-dimensionality typically seen in SEM images.

There are few prior studies applying CLSM to image arthropods based on their natural fluorescence. Galassi (1997a, b) and Galassi et al. (1998) found CLSM to be superior to compound light microscopy for fine details and used the technique for taxonomic descriptions of copepods. Zill et al. (2000) used CLSM imaging in their examination of cockroach trochanter structure for biomechanical applications. Klaus et al. (2003) extensively explored the technique for imaging genitalic structures from lower and higher Diptera, and presented a detailed methodology for using CLSM in such studies. In addition to the usefulness for imaging external structures, CLSM can also create images of soft internal structures such as muscles and ducts (Klaus et al. 2003, C. Chaboo, personal communication). It would appear that CLSM can be used for virtually any fluorescent insect structure small enough to fit within the field of view on a compound microscope, although there are real limitations on the thickness of structures that can be imaged by CLSM (Masters and Farmer 1993, Skaliora and Pagakis 2002).

In the current work, we apply CLSM to various structures in the ephydroid fly families of Camillidae, Campichoetidae, and Drosophilidae, with an emphasis on their

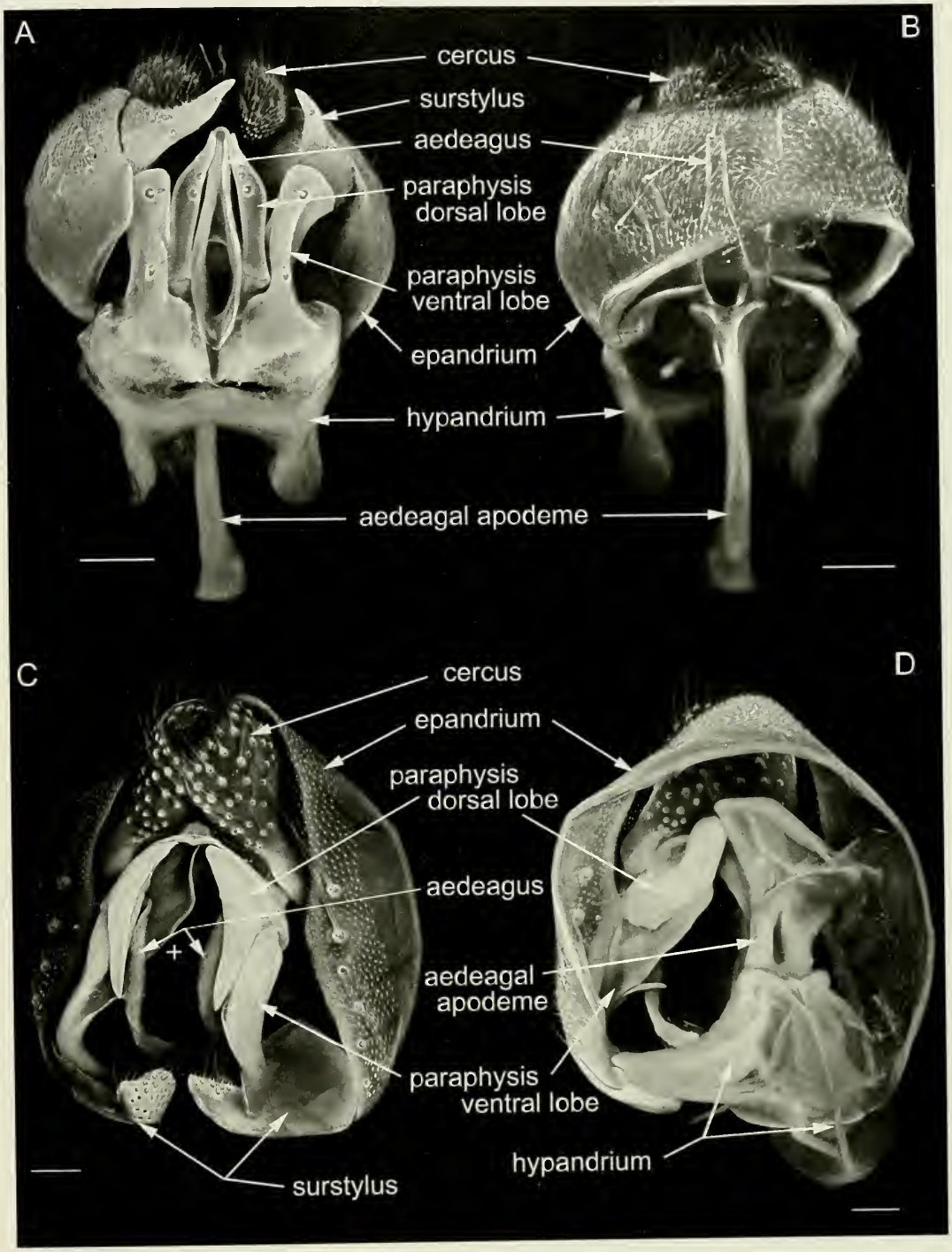

Fig. 1. CLSM maximum intensity projections for articulated male genitalia. Both ventral (A) and dorsal (B) views are pictured for *Campichoeta latigena*; (C) posterior view and (D) internal view of the genitalia of *Cladochaeta inversa*. All scale bars = 100 microns.

Table 1. Male ephydroids used in this study are from pinned specimens in the American Museum of Natural History collection and from VS dissertation.

Species	Collection Information
Cladochaeta inversa (Walker)	Houghton Co. Michigan; 20 August 1959, B. and K. Driesbach
Scaptodrosophila sp.	Australia; W. B. Mather
Afrocamilla armata Barraclough	36°10′E, 3°50′S, Tarangire NP, Tanzania, Africa. July 1994, D. Grimaldi
Campichoeta latigena McAlpine	8,700′ Rustler Park, 7 mi. W. Portal, Cochise Co. Arizona. 1 June 1991 D. Grimaldi
Drosophila parvula Bock and Wheeler	*Drosophila* Species Resource Center, Bowling Green. Stock # 14028-0621.0; 20 miles north Kuala Lumpur, Malaysia; 22–28 June 1962 Wasserman [type culture]

male terminalia. Insect male terminalia are routinely used in insect systematics for separating and defining species, and because of their complexity (Eberhard 1985) they frequently are a significant source of characters for phylogenetic studies. The complexity of the structures has also led to many ambiguities regarding homology among male genitalia in insects, including Diptera (e.g., Tuxen 1978, McAlpine 1981 vs. Griffiths 1972 vs. Cumming et al. 1995). Ephydroids are typical of the diverse array of cyclorrhaphan flies in that the male terminalia are complex as well as three-dimensional (vs. the male terminalia of nematocerous flies, which are flatter), and therefore provide a particularly appropriate subject for testing applications of CLSM.

Materials and Methods

Specimens.—The male genitalic dissections are from the species listed in Table 1. *Drosophila melanogaster* females obtained from Carolina Biological Supply Co. were used for the dissections of antenna, proboscis and female genitalia.

Dissections.—Dissections were carried out as described by Klaus et al. (2003). Dried, pointed flies were relaxed in a humidity chamber prior to dissection. For genitalic dissections, the distal half of the abdomen was removed. For the proboscis and antenna, the head was disarticulated from the thorax at the occiput. These parts were cleared by placing them in a 0.6 ml Eppendorf tube containing a 10% KOH solution and incubated for approximately 2 h in a heated water bath (70°C). The dissected parts were rinsed, partially dehydrated and transferred to glycerine through successive wells of a spot plate. The first well contained distilled water, the next two wells contained 70% ethanol, and the last well contained glycerine. Specimens were further dissected in the glycerine well.

Mounting.—Temporary slide mounts were made using glycerine jelly. Specimens were placed in a glycerine jelly droplet mounted between two cover slips (No. 1.5, nominal thickness 170 μm). Spacers measuring approximately 200 μm in thickness (i.e., two stacked pieces of No. 0 cover slips, nominal thickness 100 μm each) were placed to either side of the glycerine jelly drop containing the specimen. Mounting the specimens between two cover slips allows the specimens to be imaged from both sides by simply turning over the mount, thereby increasing the quality of image data collected. This is to avoid data loss from the side of a specimen farthest away from the sources of illumination and detection.

CLSM imaging.—Images were taken using a Zeiss 510 confocal laser scanning microscope (CLSM) equipped with an inverted Zeiss Axiovert microscope housed in the AMNH Microscopy and Imaging Facility. Specimens were imaged from both sides using a 20× Fluar dry objective lens (NA = 0.75, WD = 610 μm). In a few instances,

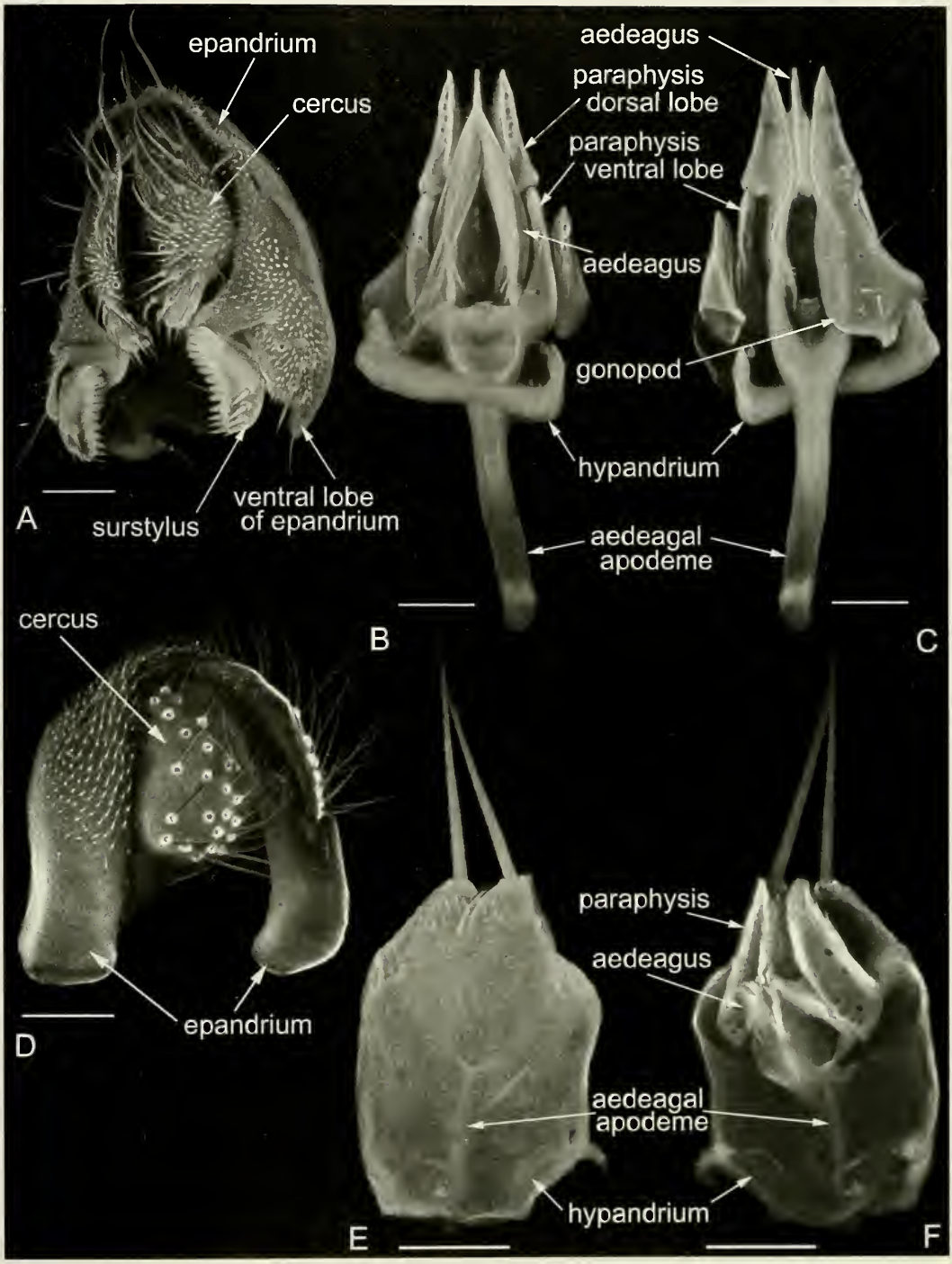

Fig. 2. CLSM maximum intensity projections of disarticulated male genitalia. An exemplar *Scaptodrosophila sp.* with a posterior view of the periphallic structures in (A) plus ventral and dorsal aspects of the phallic structures pictured in (B) and (C) respectively. Periphallic (D) posterior view and phallic (E) ventral and (F) dorsal views for *Afrocamilla armata.* Scale bars = 100 microns.

the object to be imaged (i.e., female genitalia and dorso-ventral and lateral views of the proboscis) was so large that the zoom setting was reduced to 0.70. The autofluorescence of the insect structures was induced using a helium neon laser (excitation wavelength = 543 nm) set at full power (1 mW). The fluorescence emission signal was detected using a 560 nm long pass filter; these are the factory preset filter settings for rhodamine (Cy3, Texas red) stained structures. Due to the thickness of the specimens, series of optical slice images were taken through the specimen along the z-axis. Each z-slice was 2.2 μm thick with an optimal overlapping interval of approximately 1.1 μm. Each image (or slice) was collected with the same parameters: pixel frame size of 1024 × 1024 with a unidirectional scan mode and an 8-bit pixel depth. The pixel scanning time was 6.40 μs. Every rasterized line of each frame was duplicated and the mean was taken. Image collection settings were optimized for each specimen. The amplitude gain was held constant at a value of 1.0. The amplitude offset usually equaled −0.06 (range of −0.05 to −0.07) in order to produce a maximally black background. For each specimen imaged, a single detector gain setting was used for all the z-slices in a single 3-D image stack. For each specimen the detector gain was optimized so that brightest part of the whole sample contained only a few red (oversaturation) spots. The Zeiss 510 LSM program function was used for pinhole size optimization (74 μm for all specimens).

3-D image reconstruction.—Maximum intensity projections were generated using the Zeiss 510 LSM® software version 3.2. Image contrast was enhanced using Adobe PhotoShop® version 6.0.1. In some instances the transparency setting for optical image stacks altered in the Zeiss 510 LSM® software to reveal internal structures *in situ* while obviating the destructiveness of actual (physical) dissection.

Light microscope photomicrography.—Bright field light microscope (BFLM) images were captured using the CLSM Zeiss Axiovert microscope and the software setting for transmitted light. Single frame digital images were collected at various focal planes within the specimen. All the settings were as described above, except that no emission filter was used. This method of BFLM is similar to photomicrography using a traditional bright field compound microscope, except that a laser source of light is used for illumination instead of a white light flood source. BFLM images were collected using a 20× Fluar dry objective lens (NA = 0.75, WD = 610 μm).

Morphological terminology.—Terminology used follows Grimaldi (1990) and McAlpine (1981).

Results and Discussion

Protocols developed at the AMNH provide optimal CLSM images of insect cuticle (Klaus et al. 2003). We applied those protocols here to complex structures of ephydroid fly terminalia, proboscis and antennae. Unlike SEM, these CLSM images differentiate insect structures on the basis of degree of naturally occurring autofluorescence from an undetermined molecular source(s) (see Klaus et al. 2003 for discussion). For male terminalia, CLSM images provide a refined view of structures, thus improving the ability to properly homologize structures. This is particularly important for the periphallic structures such as the paraphyses and gonopods, which flank the aedeagus. These can be elaborately developed, as in *Cladochaeta* (Fig. 1), or they can be reduced, or even lost in other drosophilids. The hypandrium (an internalized, ninth sternite) and epandrium (a capsule-like eighth tergite) are generally easily discerned, though there are lobes of each developed in various genera, and these lobes are sometimes articulated. The phallic and periphallic complex are attached to the hypandrium and lie within the epandrium, so these structures—which are routinely used for species determinations in these flies—are only partially observable with an SEM.

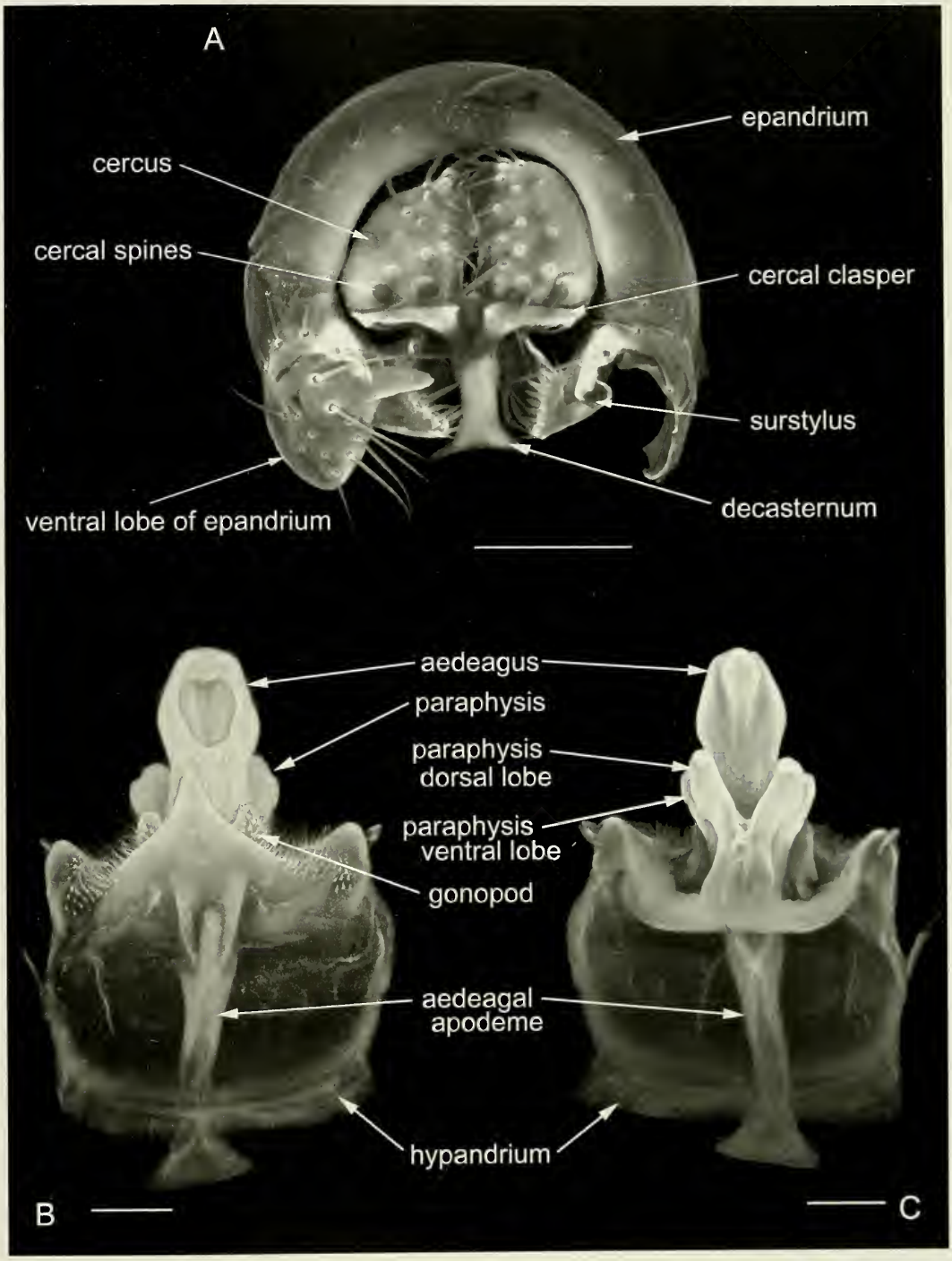

Fig. 3. Maximum intensity projection of *Drosophila parvula* male genitalia imaged with CLSM. (A) Dorsal view of the periphallic structures; (B) ventral and (C) dorsal view of the phallic structures. All scale bars = 100 microns.

Fig. 4. Bright field light microscope images of *Drosophila parvula* male phallic structures, including the phallus, paraphyses, gonopods and the pointed medial lobe in the upper portion of the hypandrium. The focal plane progresses from the ventral to the dorsal side of the structure—images (A) through (C).

CLSM revealed that each paraphysis of *Cladochaeta inversa* (Walker) is composed of a dorsal and a ventral lobe (Fig. 1), which are pressed closely against each other, but with a distinct suture between them. The paraphyses in *Cladochaeta* had previously been interpreted as a complete structure (Grimaldi and Nguyen 1999). In *Scaptodrosophila* sp. the paraphysis is also articulated, but with a small distal lobe (Fig. 2) that is most distinct in ventral view. In *Drosophila parvula* Bock and Wheeler a bilobed paraphysis is readily seen, also with a dorsal one closely adpressed over a ventral one (Fig. 3), which is a structure rarely reported or never interpreted as such before in the subgenus *Sophophora*. The setae on the paraphyses of *Afrocamilla, Campichoeta,* and *Scaptodrosophila* have wide, deep socketed bases (Figs. 1–2), similar to those of trichobothria or sensilla trichodea, and thus are probably sensory in function. The gonopods in drosophilids are a pair of structures articulating with the lateral arms of the hypandrium and lying against or over the aedeagus. Recognizing or discerning their structure is often difficult. Indeed, they are small in *D. parvula,* but are still clearly distinguished, and have scattered microtrichia. Gonopods are much larger in *Scaptodrosophila* sp., but also have fine setae. In *Campichoeta* there are two pairs of periphallic structures (Fig. 1), the inner pair of which (immediately flanking the aedeagus) could be interpreted as gonopods. However, the deep, wide sockets of the setae on both pairs of lobes (similar to what is found in *Scaptodrosophila*) indicate these are paraphyses.

Until now, scientists have had to rely on DIC optics and variation in illumination to discern subtle differences in slide-mounted, transparent insect structures. A comparision of the CLSM image for the ventral hypandrium of *D. parvula* (Fig. 3b) with the bright field image for the same structure (Fig. 4) illustrates the limitations of transmitted light microscopy. The compound light microscope shows structures, albeit

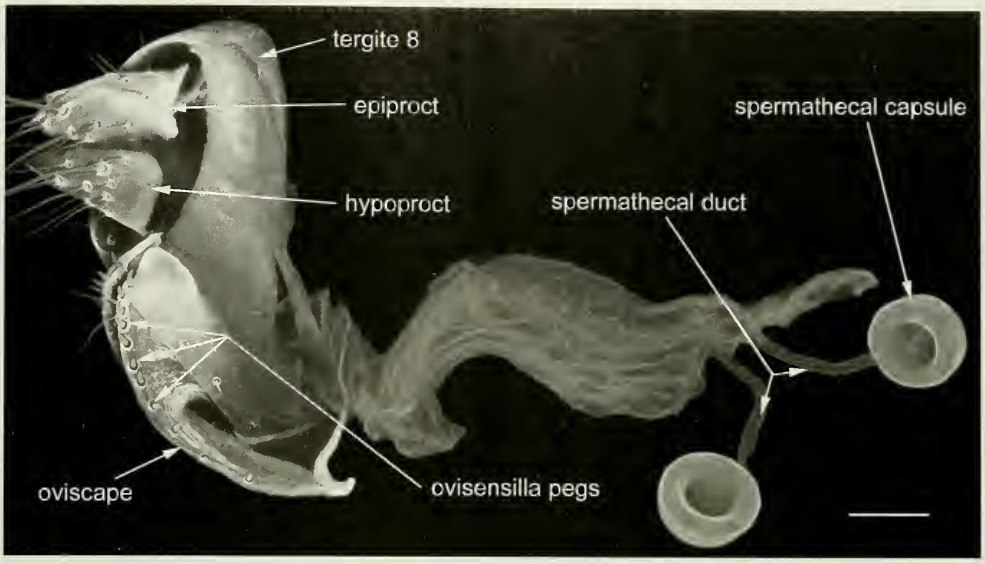

Fig. 5. Maximum intensity projection of CLSM image stack of internal and external female genitalic structures of *Drosophila melanogaster*. Note that structures range broadly in the degree of sclerotization, from heavily sclerotized to completely membranous. Scale bar = 100 microns.

blurred, simultaneously from multiple focal planes. Therefore, edges and depth cues can be confused by variation in sclerotization of structures. The CLSM, however, through the use of a pinhole, only collects in-focus images from one focal plane at a time, thus facilitating the creation of 3-D representations of an object.

There are few studies comparing the female genitalia of ephydroid flies (Throckmorton 1962, 1966; Grimaldi 1990; Grimadi and Nguyen 1999), yet female terminalia possess numerous features that are probably important for taxonomic investigations. Among ephydriod flies the female genital apparatus is composed of the terminal abdominal segments (8 and 9). Female *D. melanogaster* (Fig. 5) exhibit the apomorphic drosophilid loss of a cercus—a paired lobe structure located postero-ventral to the epiproct (tergite 9) and the hypoproct (sternite 9) in ephydriods. The 8th sternite is the lobate oviscape (also referred to as the oviscapt). Among ephydriods, the oviscape varies in overall shape. Additionally, the distribution and orientation of the setae along the margin varies, as does the type of setae (fine or pegs). The variation in the oviscape may corrolate with oviposition substrate (e.g., flowers) as well as phylogeny. The *D. melanogaster* oviscape pictured in Fig. 5 has a broad, blunt tip used to deposit eggs in soft surface of rotting fruits and possesses pegs an apomorphic condition within *Drosophila* (Grimaldi 1990). Among ephydriods, sperm storage organs are either ventral/seminal receptacle (e.g., Camichoetidae, Diastatidae, Ephydridae) or spermathecae. Both vary in overall shape, size and degree of sclerotization. The spermatheca of *D. melanogaster* is a broad capsule (Fig. 5) (pleisomorphic condition among drosophilds [Grimaldi 1990]) fairly well sclerotized with a single spermathecal duct. The duct and its articulation with the capsule are extremely difficult to discern using a light microscope, but easily visualized using CSLM (Fig. 5).

In most higher Diptera, the proboscis is used for mopping surface liquids from the substrate, which in the case of drosophilids includes a suspension of yeasts, fungi, bacteria and sugars, associated with rotting fruits or other plant parts and slime fluxes,

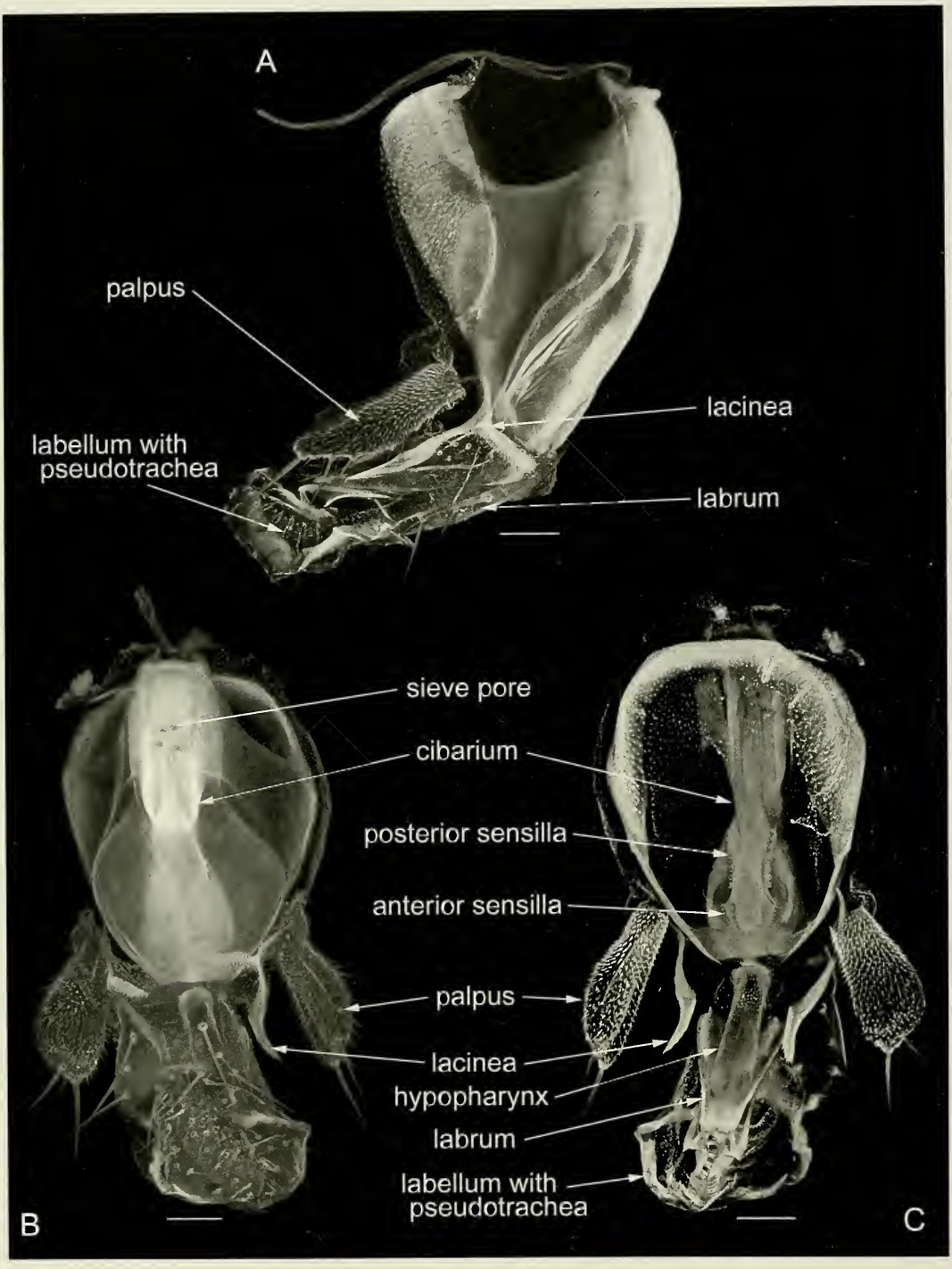

Fig. 6. Maximum intensity projection of female *Drosophila melanogaster* proboscis. (A) Lateral view; (B) and (C) dorsal and ventral views, respectively. Notice that internal structures traditionally manually dissected such as the lacinia, cibarium and hypopharynx are clearly visible, allowing *in situ* examination. Scale bars = 100 microns.

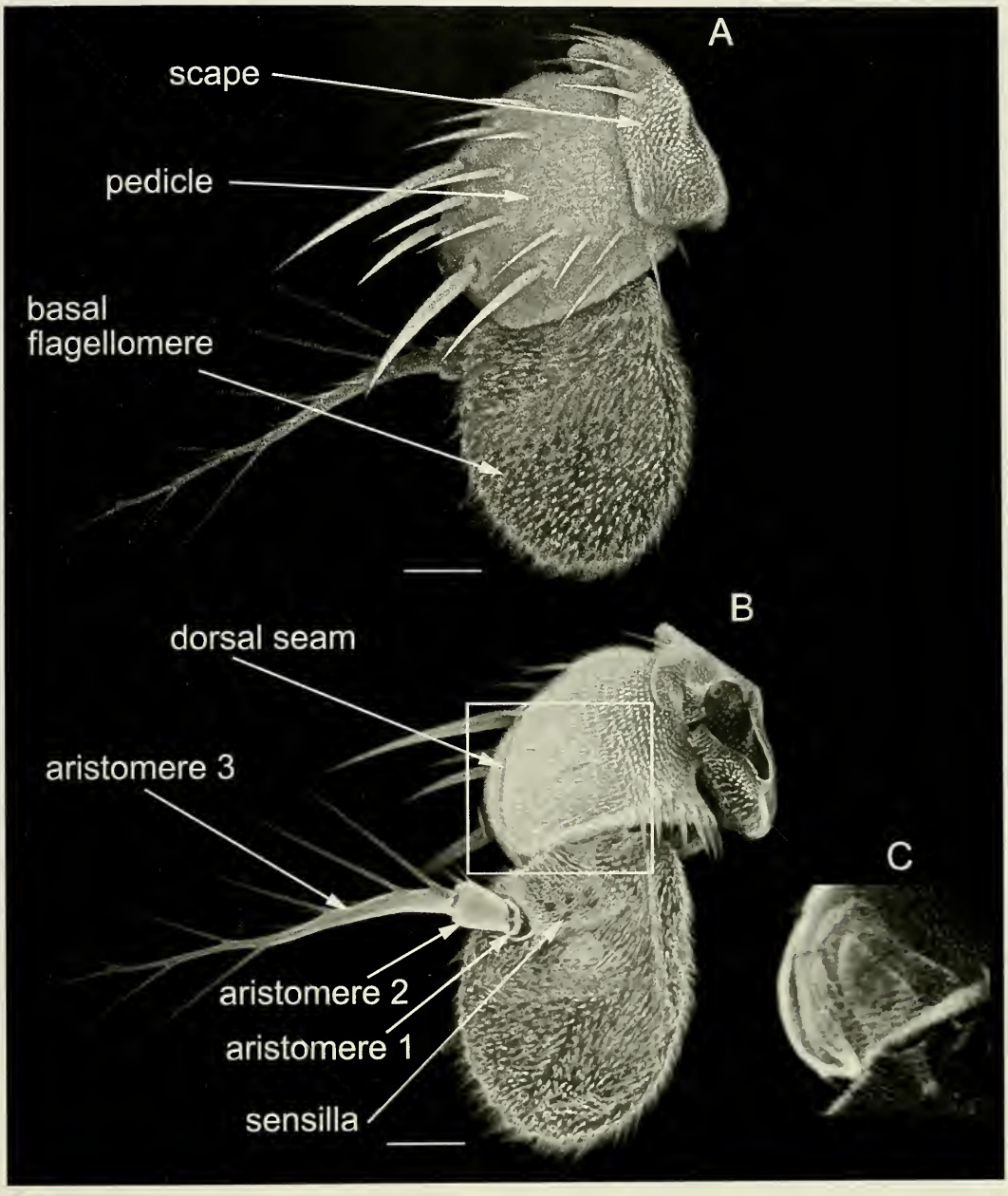

Fig. 7. Medial (A) and lateral (B) views of a female *Drosophila melanogaster* antenna imaged using CLSM. Scale bars = 100 microns. The conus was made visible in (C) by changing the transparency setting in the 3-D rendering of the maximum intensity projection. The boxed region in (B) indicates the area of the specimen that was rendered transparent.

and in a few instances pollen and nectar. The various components of the proboscis (e.g., hypopharynx, cibarium and lacinia) vary among ephydriod flies and have been used as a source of phylogenetic information (Grimaldi 1990). For illustrative purposes lateral and dorsal-ventral views of a female *D. melanogaster* proboscis is pre-

sented in Fig. 6. The shape of the hypopharnyx (tube used to conduct food) and cibarium (pump apparatus with associated muscles used to create the suction) vary diagnostically among ephydriods. The various sensilla along the cibarium (anterior, posterior and sensilla pores) vary in number and arrangement (Fig. 6) these sensilla probably function as stretch receptors. Another variable ephydriod structure is the paired laciniae, each laterally placed along the proboscis, which vary in shape and setation. Many of these minute, but phylogenetically informative, structures are embedded within the proboscis and require difficult, destructive, and time consuming dissection in order to isolate them.

Antennal surface features, especially areas of fine setation such as the sensilla on the basal flagellomere, are thoroughly documented using CLSM in Fig. 7. Additionally, by subsampling the image stack and manipulating the transparency setting in the 3-D reconstruction, internal structures, such as the conus can be isolated. The conus possesses structural variation at the family and the genus levels within drosophilids (see Grimaldi 1990).

Possible applications of CLSM for insect morphology could be substantial, particularly for the study of obscure, intricate, sclerotized structures that have been difficult to observe or interpret, including: minute sclerites in the proboscis (e.g., the hypopharynx of psocopterans and lice); the proventriculus and its array of internal spines in fleas, boreid mecopterans, Dictyoptera, and other insects; the complex of axillary sclerites at the base of the wing, and their muscular insertions; and, of course, terminalia. The expense of CLSM instruments will probably prohibit their use for routine imaging in systematics, but the implications for systematics are likewise highly significant. First, few illustrations can compare with the fidelity of a CLSM image. As a prolific illustrator, one of us (DG) acknowledges that even the best illustrations are subjective in what they do not portray (as trivial information) as in what they do. Given that the best CSLM images can also be manipulated for any view, they provide superior renditions of types and other unique specimens critical for identification. Digitally sharing of information with remote colleagues also obviates the need to ship types or other valuable specimens.

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